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PRINCIPAL INVESTIGATOR: Ruth R. Levine, Ph.D.

CONTRACTING ORGANIZATION: Boston University School of Medicine
Boston, Massachusetts 02118

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6. AUTHOR(S)
Ruth R. Levine, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Boston University School of Medicine
Boston, Massachusetts 02118

E-Mail: nwilcox@med-biochem.bu.edu

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The First International Symposium on Subtypes of Muscarinic Receptors held in 1983, promulgated the novel concept that there was more than one subtype of muscarinic receptors (mAChR). Now The Ninth Symposium is the first meeting to describe the phenotypes of mice lacking each of the five muscarinic receptor genes. These knockout (KO) data are helping to provide the rationale for some novel therapeutic targets for selective muscarinic agents and are also furthering the understanding of complex functional consequences of mAChR activation. It has become possible with the availability of knockout mice to identify which subtypes are involved in motor function control and to develop selective antagonists for Parkinson's Disease lacking the limitations of older compounds. The role of M_1 selective agonists in treating Alzheimer's Disease was discussed as well as the potential usefulness of M_2 antagonists in treating the cognitive decline associated with this disease. Results with muscarinic compounds that act at M_3 receptors suggest that selective compounds may have therapeutic efficacy in glaucoma and there is emerging evidence that an M_4 agonist could be a reasonable target for the treatment of schizophrenia. The role of selective muscarinic agents in the treatment of 1) chronic obstructive pulmonary disease; 2) acute attacks of asthma; 3) urinary incontinence and 4) GI motility were also discussed.

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NINTH SYMPOSIUM ON SUBTYPES OF MUSCARINIC RECEPTORS

FINAL REPORT

RUTH R. LEVINE

May 8, 2001

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The recently completed Ninth Symposium on Subtypes of Muscarinic Receptors upheld the standards for excellence set by the eight previous meetings. This ninth symposium was of particular historic significance since this was the first meeting at which descriptions of phenotypes of mice lacking each of the muscarinic receptor genes were available. The discussions of these knockout data in the development of some novel therapeutic targets for selective muscarinic agents were exciting but of equal import and timeliness were the lectures on receptor mechanisms and therapeutic approaches to the treatment of Parkinson's Disease, Alzheimer's Disease, glaucoma, schizophrenia, chronic obstructive pulmonary disease and urinary incontinence. These were also a large numbers of first-rate and informative posters presented. Also noteworthy were the invited participation of women in the scientific sessions, the large number of predoctoral fellows and young scientists whose attendance was made possible by funds granted to the Symposium, and the short oral presentations of selected poster papers.

There were 120 attendees at the Ninth Symposium, one-third of whom were from 14 countries outside the United States. Twenty-two (22) major papers were presented in the regular sessions, five of these by women scientists. A total of 56 poster papers were discussed in the two scheduled afternoon sessions, but posters were available for viewing for the entire 4-day meeting. Eight posters were selected for 10 minute oral presentations at an afternoon session set aside for this new feature. It is also noteworthy that the papers selected for oral presentations on the basis of their importance and impact included that of 3 predoctoral fellows, one of whom was a female, and one minority scientist; the choices were made by the Committee in the absence of any prior knowledge of the status of the authors.

The Proceedings of the Ninth Symposium, which contains the edited manuscripts of the papers presented in the regular sessions as well as the abstracts of the poster papers is to be published in Life Sciences as Volume 68, Nos. 22 and 23. Proceedings will be sent free of charge to the thousands of subscribers of Life Sciences worldwide

as well as to hundreds of others including all of our pre- and postdoctoral fellows. Grant support from the U.S.Army Medical Research Acquisition Activity was largely responsible for the publication of the Proceedings and this has been acknowledged in the program distributed to all attendees and Life Sciences.

We are extremely pleased that the grants we received and the funds contributed by 18 pharmaceutical companies made it possible for us to pay the travel and accommodation expenses for 19 speakers and committee members from academia. The figure of which we are most proud, however, concerns the large number of pre- and postdoctoral students whom we were able to bring to the Symposium. There were 21 in all and 17 of these young investigators used this opportunity to present the results of their own research. The attendance of these young scientists was made possible and was largely supported by grants from the National Institute of Neurological Disorders and Stroke and from the National Science Foundation as well as by contributions from industry. A list of pre- and postdoctoral fellows and their affiliations is appended along with samples of the many letters received indicating the benefits derived from, and the overall success of, the Symposium.

We are also very proud of the fact that the editor of Trends in Pharmacological Sciences (TIPS) solicited a report of our meeting. This report (copies of which are attached) was published in the April edition of TIPS. Mr. Adam Smith, the editor of TIPS, had glowing comments on the report submitted by Drs. Birdsall, Nathanson and Schwarz, e.g.: "a full and exciting meeting report" having "the flavour of a fast-expanding field." The article as submitted was longer than the standard review but Mr. Smith did not wish it shortened because "the material is going to be too valuable to too many readers."

Another measure of success of the Symposium was the high degree of active participation of the registrants in the formal sessions of the Symposium and their interaction in informal gatherings. The attendance at each of the five lecture sessions was excellent and appeared to include all registrants. The 5-10 minute period of discussion between papers was informative and provocative. The excellent lighting and space available for posters encouraged lengthy discussions by both presenters and viewers. There appeared to be much enthusiasm for the research being presented particularly by the young scientists. All in all, it is evident that the Ninth Symposium on Subtypes of Muscarinic Receptors has not only encouraged the research efforts of young investigators but has also stimulated and is stimulating additional research which should, indeed, lead to the development of new and better therapeutic

agents as well as agents useful to the U.S.Army Research and Acquisition Activity and to the U.S.Army Medical Research Institute Chemical Defense.

Plans for the Tenth Symposium have been set in motion. The Symposium would be held in the year 2002, this time again in conjunction with the meeting of the Society for Neuroscience. We hope that USAMRAA will again help to support the publication of the Symposium's Proceedings.

Pre- and Postdoctoral Fellows Supported by Symposium

Predoctoral Fellows

1. Mark Bee
   National Institute for Medical Research
   London, United Kingdom

2. Jennifer Berkeley
   Dept. of Neurology
   Emory University
   Atlnata, GA

3. Gordon Buchanan
   Dept. of Molecular and Integrative Physiology
   University of Illinois at Urbana-Champaign
   Urbana, IL

4. Stefan Buller
   Dept. of Psychiatry
   Penn State University
   Hershey, PA

5. Yang Cao
   Dept. of Medicinal & Biological Chemistry
   University of Toledo
   Toledo, OH

6. Isolde Erlenbach
   Laboratory of Bioorganic Chemistry
   NIH-NIDDK
   Bethesda, MD

7. Christopher Evans
   Johns Hopkins University
   Baltimore, MD

8. Gina Forster
   Dept. of Psychology
   Macquarie University
   Sydney, Australia

9. George Laszlo
   Dept. of Pharmacology
   University of Washington
   Seattle, WA

10. S. Julie Ngola
    Dept. of Pharmacology & Toxicology
    Wright State University School of Medicine
    Dayton, OH

11. Paul Park
    University of Toronto
    Faculty of Pharmacy
    Toronto, Canada

12. Diane Porter
    Dept. of Pharmacology
    Penn State University College of Medicine
    Hershey, PA

13. Darakh-Shanda Shehnaz
    University of California
    Irvine, CA

14. Jian-Bing Shen
    Dept. of Pharmacology
    University of Connecticut Health Center
    Farmington, CT

15. Laura Volpicelli
    Emory University
    Atlanta, GA

Postdoctoral Fellows

1. Moritz Bünemann
   Dept. of Pharmacology
   University of Wuerzburg
   Wuerzburg, Germany

2. Fiona Coulson
   Johns Hopkins University
   School of Public Health
   Baltimore, MD

3. Jing-Sheng Liang
   Dept. Molecular Pharmacology
   University of Miami School of Medicine
   Miami, FL

4. Abulkhair Mamoon
   Dept. of Pharmacology & Toxicology
   University of Mississippi Medical Center
   Jackson, MS

5. Liping Mou
   Neuroscience Institution
   Morehouse School of Medicine
   Atlanta, GA

6. Craig Weiss
   Dept. Cell & Molec. Biol.
   Northwestern University Medical School
   Chicago, IL

improvements in primate well-being along with increased emphasis on pain-free and non-invasive investigations.

To progress responsibly, on-going international conversations involving all the participants (i.e. the public, patients and their families, physicians, veterinarians and others concerned about animal welfare, ethicists and religious leaders, governmental officials and others knowledgeable in regulatory issues, and scientists) must continue and be enriched. Perhaps the time has come to convene a National Advisory committee of experts to solicit comments and then chart directives on the best course of contribution of genetically modified and/or identical non-human primates for accelerating cures and discoveries in molecular medicine. None of us knows where this field will be 50 or even 10 years from today – however, we can all be faulted if the difficult challenges raised by Dunnett are not addressed soberly.

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Anthony W.S. Chan
Kowit-yu Chong
Diana Takahashi
Crista Martinovich
Nancy Duncan
Oregon Regional Primate Research Center
Laura Hewitson
Calvin Simerly
Oregon Regional Primate Research Center and Dept of Obstetrics-Gynecology
Gerald Schatten*
Oregon Regional Primate Research Center and Depts of Cell-Developmental Biology and Obstetrics-Gynecology, Oregon Health Sciences University, 505 NW 185th Avenue, Beaverton, OR 97006, USA.
*e-mail: schatten@ohsu.edu

Meeting Report

Muscarinic receptors: it's a knockout

Nigel J.M. Birdsall, Neil M. Nathanson and Roy D. Schwarz

The *Ninth International Symposium on Subtypes of Muscarinic Receptors was held on 31 October - 4 November 2000 in Houston, Texas, USA.**

When the *First International Symposium on Subtypes of Muscarinic Receptors* was held in 1983, a key purpose of the meeting was to promulgate the novel concept that there was more than one subtype of muscarinic acetylcholine receptor (mAChR).

The *Ninth International Symposium on Subtypes of Muscarinic Receptors* was a particularly historic occasion because this was the first meeting at which descriptions of the phenotypes of mice lacking each of the five mAChR genes were available. A summary of some of the newer data, together with older published data[1], on the properties of mAChR knockout (KO) mice is shown in Table 1. These KO data are helping to provide the rationale for some novel therapeutic targets for selective muscarinic agents and are also furthering the understanding of the complex downstream functional consequences of mAChR activation. Another timely topic that emerged at this meeting was the potential to use the high-resolution structure of rhodopsin[2] to model the mAChR (and other related receptors).

*The proceedings of the meeting will be published: Birdsall, N.J.M. *et al. Life Sciences* (Vol. 68).

Receptor knockouts

The role of the muscarinic M_1 receptor in signal transduction in cultured cerebral cortical neurons was described by Neil Nathanson (University of Washington, Seattle, WA, USA). Activation of both the phospholipase C (PLC) and the mitogen-activated protein kinase (MAPK) cascades by mAChR agonists was greatly impaired in cultures from M_1 receptor KO mice compared with wild-type (WT) controls. Consistent with the M_1 receptor being the main subtype coupled to the G_q family of G proteins in the forebrain, Frank Bymaster (Lilly Research Laboratories, Indianapolis, IN, USA) reported that, in hippocampal membranes from M_1 receptor KO mice, there was a loss of agonist-stimulated GTPγ^{35}S binding to $G_q\alpha$.

Data from a large number of laboratories have implicated MAPK in neuroplasticity, memory and learning. Consistent with this hypothesis, Nathanson reported that the M_1 receptor KO mice exhibited decreased performance in a hippocampal-based test of spatial learning and decreased long-term potentiation (LTP) at the Schaffer-collateral–CA1 synapse in the hippocampus. These results support those obtained in previous pharmacological studies on the role of the M_1 receptor in memory and learning, and increase the hope that a selective M_1 receptor agonist might alleviate the cognitive deficits in patients with early stages of Alzheimer's disease.

By contrast, there have been conflicting reports on the potential role of M_1 receptors in the heart, using different species. Nathanson reported that both biochemical and physiological studies did not support the presence of the M_1 receptor in murine heart, although activation of M_1 receptors in the sympathetic ganglia can cause catecholamine-mediated cardiac stimulation.

Martha Gillette (University of Illinois, Urbana, IL, USA) described the role of the

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Table 1. Properties of muscarinic receptor knockout mice[a]

Receptor	Phenotypes or responses lost in knockout
M_1	Decreased activation of PLC and MAPK in the forebrain, loss of pilocarpine-induced seizures, reduced hippocampal-based memory and learning, reduced LTP at Schaffer-collateral–CA1 synapses, loss of regulation of M-current K^+ channel in the SCG, loss of slow regulation of Ca^{2+} channels in the SCG, loss of agonist-mediated circadian rhythm phase advance
M_2	Loss of agonist-induced bradycardia, major pathway for mAChR-mediated analgesia, major pathway for mAChR-mediated hypothermia, loss of oxotremorine-induced tremor, loss of autoreceptor-mediated regulation of ACh release, loss of fast regulation of Ca^{2+} channels in the SCG
M_3	Decreased mAChR-mediated salivation, decreased mAChR-mediated pupillary constriction, decreased body and fat pad weight, decreased plasma leptin concentrations, decreased mAChR-mediated smooth muscle contraction *in vitro*, urinary retention and bladder distension
M_4	Increased basal locomotor activity, increased dopamine D1-receptor-mediated locomotor activity, minor pathway for mAChR-mediated analgesia
M_5	Minor (slow) pathway for mAChR-mediated salivation, increased drinking after water deprivation, large decrease in mAChR-mediated dopamine release

[a]Abbreviations: ACh, acetylcholine; LTP, long-term potentiation; mAChR, muscarinic acetylcholine receptor; MAPK, mitogen-activated protein kinase; PLC, phospholipase C; SCG, superior cervical ganglion.

M_1 receptor in *in vivo* circadian rhythm behavior and electrical activity of the suprachiasmic nucleus (SCN) in isolated brain slices. *In vivo* administration of mAChR agonists into the SCN caused a phase advance in the circadian rhythm of wheel-running activity in WT mice, but not in M_1 receptor KO mice. mAChR agonists, when applied during subjective night but not subjective day, also caused a large phase advance in electrical activity recorded from SCN slices, an effect that is lost in M_1 receptor KO mice. Pharmacological and biochemical analyses of this response are consistent with a M_1-receptor-mediated, cGMP-dependent protein kinase pathway.

Jurgen Wess (National Institutes of Health, Bethesda, MD, USA) had previously reported that, although M_2 receptor KO mice exhibited a significant impairment of mAChR-mediated analgesia, there was no alteration in analgesia in the M_4 receptor KO mice. However, Wess showed that the M_4 receptor does have a minor role in this response because the small amount of analgesia remaining in the M_2 receptor KO was completely lost in M_2–M_4 receptor double KO mice. Consistent with pharmacological analyses, both Bymaster and Wess found that the M_2 receptor was the main autoreceptor in the brain because mAChR-mediated regulation of acetylcholine (ACh) release was greatly reduced in the M_2 receptor KO mice.

Both Wess and Minoru Matsui (University of Tokyo, Japan) described the generation and analysis of M_3 receptor KO mice. These animals displayed decreased pupillary constriction to both light and agonist treatment, significant urinary retention and bladder distension (although this was much less pronounced in females) and decreased contraction of urinary tract muscles. Although the gastrointestinal tract appeared normal in these animals, there was decreased contractility of ileal smooth muscle *in vitro*. However, some contrasting results were obtained in M_3 receptor KO mice. Both Matsui and Bymaster (using mice from Wess) reported decreased salivation, whereas Wess reported unaltered salivation. Although Matsui found that there was decreased body weight when mice were fed dry but not wet food, Wess reported decreased food consumption and decreased body weight with both diets; this decreased body weight was associated with a decreased mass of peripheral fat pads. Wess also reported a large decrease in the concentration of leptin, suggesting a previously unsuspected role of the M_3 receptor in the action of this factor.

John Yeomans (University of Toronto, Canada) described the effects of targeted disruption of the M_5 receptor. These KO mice exhibited decreased salivation at relatively long times after administration of agonists, and drank more water after water deprivation than WT mice. Most dramatically, after stimulation of mesopontine ACh-containing neurons a large decrease in dopamine release was observed in the nucleus accumbens, which is consistent with a role for M_5 receptors in the ventral tegmental area in the rewarding effects of hypothalamic stimulation.

The role of the various mAChR subtypes in electrophysiological responses in neurons from the superior cervical ganglia was described by Marc Shapiro (University of Washington, Seattle, WA, USA). Regulation of the M-current K^+ channel was completely lost only in the M_1 receptor KO mice, which indicates that the M_1 receptor was the only subtype that regulated this channel in sympathetic neurons. There are two pathways for the inhibition of voltage-sensitive Ca^{2+} channels in sympathetic neurons, the slow, voltage-independent inhibition of L-, N- and P/Q-type channels and the fast, membrane-delimited, voltage-dependent pathway. The former was completely lost in neurons from the M_1 receptor KO mice and the latter was absent in M_2 receptor KOs but normal in M_4 receptor KO mice. Interestingly, this result contrasts with pharmacological and molecular biological studies that indicate that the M_4, and not the M_2, receptor mediates this response in rats. This difference demonstrates that, although studies with gene-targeted mice can provide a wealth of useful information, the physiological role of a given receptor subtype can vary between species.

Therapeutic approaches

Parkinson's disease

Anticholinergic therapy has been used historically to treat tremors and other symptoms associated with early stage Parkinson's disease. However, existing antagonists are nonselective and produce side-effects that limit their effectiveness. With the availability of the KO mice, it has become possible to identify which subtypes of mAChRs are involved in the control of motor function and to develop selective antagonists for use in Parkinson's disease that overcome the limitations of older compounds.

John Salamone (University of Connecticut, Storrs, CT, USA) described work in rodents showing that non-directed, chewing-like movements produced by cholinomimetics have many of the characteristics of parkinsonian tremor. Evidence from his laboratory points to activation of M_4 receptors as being crucial for the induction of these vacuous (or tremulous) jaw movements. Additionally, M_4 receptor KO mice are hyperactive and resistant to the induction

of tremor. Thus, an M_4 receptor antagonist could prove useful as an antiparkinsonian drug. In this context, Roy Schwarz (Pfizer, Ann Arbor, MI, USA) described a series of benzoxazine isoquinolines that are selective M_4 receptor antagonists.

Alzheimer's disease

Based on the ACh hypothesis of aging and dementia, it has been suggested that acetylcholinesterase (AChE) inhibitors, direct-acting mAChR and nicotinic receptor agonists, allosteric modulators and ACh-releasing agents would be useful in the symptomatic treatment of Alzheimer's disease. AChE inhibitors have shown clear therapeutic utility, whereas convincing efficacy data for the other drug classes are lacking at present.

Alexander Walland (Boehringer Ingelheim, Germany) described the pharmacodynamic profile of the selective M_1 receptor agonist talsaclidine in both animals and humans. In isolated tissue preparations, talsaclidine showed a selectivity for M_1 receptors relative to sabcomeline, which had activity at both M_1 and M_3 receptors. Translating these results to the *in vivo* situation, talsaclidine, in contrast to sabcomeline, produced very little bronchospastic activity in guinea-pigs following intravenous administration. In clinical safety trials, talsaclidine was well tolerated in both healthy volunteers and Alzheimer's disease patients following acute and multiple dosing.

As an alternative to mAChR agonists for the treatment of Alzheimer's disease, work has focused on compounds that increase ACh release. Because muscarinic autoreceptors appear to be of the M_2 subtype, a selective M_2 receptor antagonist would increase ACh release and make more neurotransmitter available to activate muscarinic and nicotinic postsynaptic receptors. This latter action would distinguish them from pure mAChR agonists and make this therapeutic class more like AChE inhibitors at the level of the synapse.

Jean Lachowicz (Schering-Plough Research Institute, Kenilworth, NJ, USA) described the high M_2 receptor selectivity of SCH72788, which has a reasonable *in vivo* activity and, in conscious rats, increases ACh concentrations in the striatum and shows positive effects in a rat model of passive avoidance. These results suggest that M_2 receptor antagonists might be useful in treating the cognitive decline associated with Alzheimer's disease.

Glaucoma

Glaucoma is a disease of the optic nerve as evidenced by a loss of the visual field, and appears to involve a loss of the trabecular network that controls fluid outflow. Daniel Gil (Allergan, Irvine, CA, USA) presented the pathophysiology of glaucoma and the rationale for the use of mAChR agonists in the treatment of this disease. Pilocarpine, one of the oldest drugs to be used in the treatment of glaucoma, increases the outflow of aqueous humor, thereby reducing intraocular pressure. However, this drug has a short duration of action and can cause night blindness in addition to impairing distance vision.

Gil described a muscarinic compound with little activity at M_3 receptors, which are present in the iris sphincter and ciliary muscle. This compound decreases intraocular pressure in monkeys with no change in pupil diameter and no effect on accommodation. These results suggest that compounds that are selective for a specific mAChR subtype might have an improved therapeutic profile in glaucoma compared with traditional mAChR agonists.

Schizophrenia

Work relating to the use of mAChR agonists in the treatment of schizophrenia was presented by Bymaster. Dopamine D2 receptor antagonists have traditionally been used to treat schizophrenia. However, other neurotransmitter systems such as those of ACh and glutamate have been implicated in the disease.

Physostigmine was shown to reduce delusions in Alzheimer's disease patients, whereas the mAChR antagonist scopolamine produced hallucinations in normal individuals. Additionally, the atypical antipsychotic clozapine has activity as an M_4 receptor agonist. During clinical studies with the mAChR agonist xanomeline it was observed that non-cognitive behaviors were positively affected and that certain individuals were less agitated and had fewer hallucinations than those given placebo.

In rats it was found that xanomeline increased the concentration of dopamine in the prefrontal cortex and decreased the spontaneous firing of dopamine A10 neurons, similar to that observed following atypical antipsychotics. Behaviorally, xanomeline inhibited conditioned avoidance, inhibited phencyclidine (PCP)-induced hyperlocomotion and produced positive effects in the eight-arm radial maze without inducing catalepsy.

In M_4 receptor KO mice, the psychotomimetic agent PCP disrupts pre-pulse inhibition to a greater degree than it does in WT mice. This suggests that there might be hyperresponsive dopamine systems as a consequence of the loss of M_4 receptors. Thus, emerging evidence suggests that an M_4 receptor agonist could be a reasonable target for the treatment of schizophrenia.

Chronic obstructive pulmonary disease

Antimuscarinics have an established role in the treatment of both upper and lower airway diseases, including bronchodilator therapy for chronic obstructive pulmonary disease (COPD), chronic bronchitis and emphysema. In clinical trials described by Bernd Disse (Boehringer Ingelheim, Germany), a new mAChR antagonist, tiotropium, inhaled once daily, has provided more than 24 h of stable bronchodilation, which was sustained over a one-year treatment period. This drug shows a selectivity for M_3 receptors that is based on its very slow dissociation from M_3 receptors ($t_{1/2}$ ~35 h) relative to that from M_2 receptors.

Inhibition by mAChR antagonists of pro-inflammatory effects [e.g. inhibition of 5-HETE (5-hydroxy-5,8,11,14-eicosatetraenoic acid) release from epithelial cells and inhibition of release of neutrophil and eosinophil chemotactic activity from alveolar macrophages] might also occur. The elucidation of this anti-inflammatory potential is an interesting target for future therapeutic research.

David Jacoby (Johns Hopkins University, Baltimore, MD, USA) provided an overview of the parasympathetic control of airway smooth muscle and discussed current anticholinergic treatments available for asthma, COPD and rhinitis. Both M_2 and M_3 receptors are found in bronchial smooth muscle and, within the lung itself, the greatest density of M_1 and M_2 receptors is found in parasympathetic ganglia. Dysfunctional inhibitory M_2 receptors on vagal nerve endings might contribute to increased ACh release resulting in reflex bronchonconstriction. The use of M_3 receptor antagonists to block this latter effect might be of particular use in COPD.

In acute asthma attacks, anticholinergics are widely used; however, in chronic stable asthma, β-adrenoceptor agonists are preferred. The 'triggers' for acute attacks are the focus of several studies, with viral infections and allergens being shown to be major factors in acute episodes. In both cases, reflex vagally mediated bronchoconstriction is involved that is sensitive to mAChR antagonist treatment.

Urinary incontinence and gastrointestinal motility

Lisbeth Nilvebrant (Bioventia Life Science Consultants, Bromma, Sweden) described the use of tolterodine for the treatment of the overactive bladder, which is a chronic condition characterized by the symptoms of frequent and urgent urination, with or without urge incontinence. Although tolterodine is a nonselective mAChR antagonist *in vitro*, it appears paradoxically to show a wide separation between activity at bladder mAChRs and those in salivary glands *in vivo* in both animals and humans. Clinical results show that the efficacy and safety of tolterodine are equal to that of oxybutynin, but that it is significantly better tolerated in adult patients. Children and the elderly also tolerate the drug very well; however, it has not been approved for pediatric use at this time.

The topic of gastrointestinal motility and cholinergic function was covered by Richard Eglen (DiscoveR_x, Fremont, CA, USA). Smooth muscle contains a heterogeneous population of M_2 and M_3 receptors with muscle contraction being predominantly associated with M_3 receptors even though there are higher numbers of M_2 receptors present in the tissue. Although M_3 receptors directly cause contraction, M_2 receptors appear to decrease sympathetically mediated relaxation by opposing elevations in cAMP and reversing the opening of K^+ channels by β-adrenoceptors. Additionally, M_2 receptors open nonselective cation channels that facilitate entry of extracellular Na^+ ions, which in turn increase Ca^{2+} entry via L-type channels. This latter action is synergistic with the release of Ca^{2+} from intracellular sites activated by M_3 receptors.

Models of the mAChR structure

The publication last summer of the first high resolution X-ray structure of the G-protein-coupled receptor (GPCR) rhodopsin[2] has provided a framework for modeling mAChRs. These receptors have been investigated intensively using mutagenesis studies. This large body of data provides a rigorous test of whether it is possible to thread the sequence of a related GPCR onto the rhodopsin structure and obtain an energy-minimized structure that can rationalize and interpret the effects on binding and function of the mutations.

Ed Hulme (National Institute for Medical Research, London, UK) has performed alanine scanning mutagenesis studies of the residues in the second, third, fourth and seventh transmembrane (TM) domains of M_1 receptors in addition to those regions of TM5 and TM6 that are thought to encompass the binding site for ACh and competitive receptor antagonists. The ACh binding site appears to correspond very closely with the retinal binding site in rhodopsin. Most of the postulated contact residues identified by the M_1 receptor mutation studies correspond to equivalent retinal contact residues in rhodopsin. These ACh contact residues, together with identified intramolecular hydrogen bonds and Van der Waals interactions, are crucial for forming the activated state of the receptor. The conformational change is postulated to involve a rearrangement of the H-bonding network to create a rotation and outward movement of TM6. This creates a pocket within the TM helices and thereby allows the C-terminal helix of the α-subunit of the G protein to bind to the receptor and initiate GDP–GTP exchange. The footprint of the intracellular face of the receptor is of a sufficiently large area to also allow interactions with the β- and γ-subunits of the G protein, which is of considerable interest because the interactions of other proteins (e.g. receptor kinases and arrestins) with receptors are promoted by βγ-subunits.

The important role of TM3 and TM6 of mAChRs in regulating receptor activity, particularly constitutive activity, was emphasized by Tracy Spalding (Acadia Pharmaceuticals, San Diego, CA, USA). These conclusions were based on the results of multiple random mutations in TM5, TM6, the second intracellular loop (i2) and regions of i3 proximal to TM5 and TM6 in M_5 receptors. In addition, the mutagenesis results of Hulme on TM3 of the M_1 receptor were used. It was postulated that there is a cluster of residues in TM3 and TM6 responsible for maintaining the receptor in an inactive state – the equivalent region in rhodopsin undergoes a conformational change on receptor activation.

Spalding also described an agonist that exhibits M_1 receptor functional selectivity. Using receptor chimeras, the epitopes important for its binding were located at the extracellular face of TM1 and TM7 of the M_1 receptor. In addition, several amino acids found to be important for ACh binding were also not important for the binding of this agonist; a similar finding for McNA343 binding was reported by Hulme. One possible interpretation of these data is that there is a second site from which mAChRs can be activated, as suggested several years ago for the allosteric actions of McNA343 (Ref. 3).

Nigel Birdsall (National Institute for Medical Research, London, UK) used the same model of the M_1 receptor to show that residues, demonstrated to be important for the binding and allosteric actions of gallamine and strychnine, were clustered together within and to one side of a cleft in the extracellular face of the receptor (Fig. 1). This might represent the location of the allosteric binding site for gallamine and be on the access route of ACh to its binding site.

The characterization of a second allosteric site on mAChRs was also described. The protein kinase inhibitor KT5720, which is structurally related to staurosporine, binds to this site and is an allosteric enhancer of ACh binding at M_1 receptors. It is possible to demonstrate the simultaneous presence of three small ligands bound to mAChRs. The location of the second allosteric site is not known at present.

Muscarinic snake toxins

The very potent snake toxin m1-toxin1 (also called MT7), which binds specifically to M_1 receptors, was described by Lincoln Potter (University of Miami, FL, USA). This toxin binds essentially irreversibly, and allosterically or sterically hinders access of an antagonist and an agonist to their binding sites. Using mutated m1-toxin1, the interaction of the toxin with the extracellular face of the M_1 receptor is beginning to be explored. The m1-toxin1 has also been used to produce an acute and focal blockade of M_1 receptors in the striatum and to investigate the roles of M_1 and M_4 receptors in controlling movement.

Receptor mechanisms

There is a convenient but deceptively simple way of remembering the preferred coupling mechanisms of the mAChR

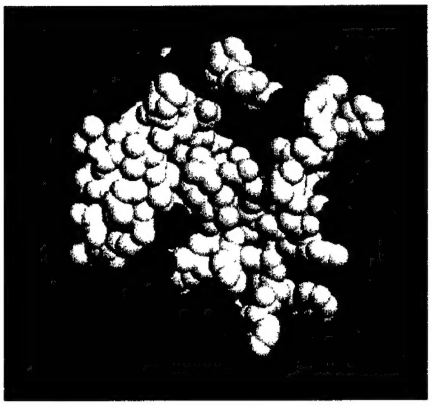

Fig. 1. View of the extracellular face of a preliminary model of the muscarinic acetylcholine M_1 receptor, based on the X-ray structure of rhodopsin. The conserved Trp400 (dark blue) at the top of transmembrane (TM) 7, important for the binding of the allosteric ligands such as gallamine and strychnine, has non-conserved residues (green) in the vicinity that have been identified as important for the observed subtype selectivity of gallamine. The clustering of these residues suggests that this is the location of the allosteric site that binds gallamine and that it appears to be associated with a cleft leading to acetylcholine (cyan), barely visible, in its binding site. The TM helices are shown in pink, the residues in the extracellular domains that are conserved across all five receptor subtypes are shown in yellow, and the non-conserved residues in the extracellular domains are shown in white. The N-terminal residues are not shown. TM1 is at the top left of the figure and TM4 is at the bottom.

subtypes. Odd numbered subtypes activate $G_{q/11}$ α-subunits that couple to PLC and generate both inositol (1,4,5)-trisphosphate [Ins(1,4,5)P_3] {and thence an increase in $[Ca^{2+}]_i$} and diacylglycerol (which can activate PKC). By contrast, the even numbered receptors activate $G_{i/o}$ α-subunits, which results in inhibition of adenylyl cyclase or opening of a class of inwardly rectifying K^+ channels (GIRKs). However, as usual, the real world is more complex: each subtype is capable of activating more than one G protein, both the Gα–GTP and the βγ-subunits generated have activities, and multiple effectors are regulated by these species[4–6].

M_2 and M_4 receptors (and other GPCRs) both inhibit and stimulate the synthesis of cAMP in a single cell line. The overall response, mediated by G_i and G_s activation, depends on agonist concentration, receptor expression level, desensitization and several additional variables. To simulate some of these phenomena in Chinese hamster ovary (CHO) cells, Stanislav Tucek (Academy of Sciences, Prague, Czech Republic) has generated a simplified model, where the observed accumulation of cAMP is represented by a basal activity multiplied by factors that represent occupancy of adenylyl cyclase type VI by G_i–GTP and stimulation of type VII cyclase by G_s–GTP, respectively.

An amplifying system for Ca^{2+} signaling by both M_2 and M_3 receptors and for other GPCRs was described by Chris van Koppen (University of Essen, Germany. Following initial production of Ins(1,4,5)P_3 by receptor-mediated PLC activation, a local discrete increase in $[Ca^{2+}]_i$ induces a rapid stimulation of sphingosine kinase to generate sphingosine-1-phosphate and eventually a 'full' Ca^{2+} mobilization.

Moritz Bünemann (University of Würzburg, Germany) described a complex example of receptor crosstalk that is mediated by M_2 and M_4 receptors but not by adenosine A_1 receptors or α_2-adrenoceptors, which also use G_i-linked pathways. Activation of M_2 and M_4 receptors, but not A_1 receptors, results in homologous desensitization of activated GIRK currents. There is also a heterologous inhibition of A_1-receptor- or α_2-adrenoceptor-activated GIRK currents by M_2 or M_4 receptors. This reversible membrane-delimited pathway is pertussis toxin insensitive, is disinhibited by exogenous Gβγ, is independent of internalization or receptor phosphorylation, and occurs downstream of G-protein activation. Interestingly, the activation of certain G_q-coupled receptors, including the M_3 receptor, can also inhibit A_1-receptor-linked GIRK channels with a similar time-course of inhibition and recovery to that exhibited by the M_2 receptor. However, the G_q pathway, but not the M_2 receptor pathway, is blocked by *Pasturella multocida* toxin (which uncouples G_q from its linked receptors), indicating that there are probably two pathways for inhibition of the GIRK current.

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Chemical names

KT5720: hexyl 2,3,9*S*,10*R*,11,12*R*-hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1*H*-diindolo[1,2,3-*fg*:3′,2′,1′-*kl*]pyrrolo[3,4-*i*][1,6]benzo-diazocine-10-carboxylate

McNA343: 4-(*N*-[3-chlorophenyl]carbamoyloxy)-2-butynyltrimethylammonium chloride

SCH72788: 4-[4-[1(*S*)-[4-[(1,3-benzodioxol-5-yl)sulfonyl]phenyl]ethyl]-3(*R*)-methyl-1-piperazinyl]-4-methyl-1-(propylsulfonyl)piperidine

Key conference outcomes

- Gene knockout experiments show discrete functions for each of the five muscarinic acetylcholine (ACh) receptor subtypes.
- Novel muscarinic agents are being developed (or have been developed) for several indications, both classical [e.g. parkinsonism, glaucoma, chronic obstructive pulmonary disease (COPD), urinary incontinence and acute asthma attacks] and newer areas (e.g. Alzheimer's disease, schizophrenia, gastrointestinal motility, irritable bowel syndrome and analgesia).
- The properties of the knockout mice point to additional potential therapeutic areas.
- The structure of rhodopsin provides a reasonable basis for modeling the ACh binding site on muscarinic receptors and possibly other binding sites on the receptor.
- An amplifying system for Ca^{2+} signaling by G-protein-coupled receptors (GPCRs) and pathways for receptor crosstalk resulting in inhibition of GIRK currents were reported.

Nigel J.M. Birdsall*
Division of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London, UK NW7 1AA.
*e-mail: nbirdsa@nimr.mrc.ac.uk
Neil M. Nathanson
Dept of Pharmacology, Room K536A Health Sciences Building, University of Washington, Box 357750, Seattle, WA 98195-7750, USA.
Roy D. Schwarz
Pfizer Global Research & Development, Ann Arbor Laboratories, CNS Pharmacology, 2800 Plymouth Road, Ann Arbor, MI 48105, USA.